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Simultaneous determination of eight corticosteroids in bovine tissues using liquid chromatography–tandem mass spectrometry

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ABSTRACT

This paper describes a newly developed method for the simultaneous determination of eight corticosteroid residues in bovine muscle, liver and kidney samples using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The determination of methylprednisone, the main metabolite of methylprednisolone, in bovine tissues using LC–MS/MS is carried out for the first time. The method development demonstrates that the pH is important in optimizing the sample preparation. Tests performed using different solid-phase extraction (SPE) cartridges were enabled to produce conditions for reducing the matrix effects (ion suppression and enhancement) of analysis. Acidic condition and mixed-mode cation exchange SPE columns resulted in the most suitable clean-up for muscle and liver, and also yielded acceptable results for kidney. The enhanced sample clean-up resulted in excellent clear baselines of ion transitions, and therefore, a higher delta electron multiplier voltage (ΔEMV) could be set in the MS/MS detector. The application of 500 V of ΔEMV improved the signal responses, however, the noise level did not change, and consequently, the overall sensitivity and analytical limits (limit of detection, limit of quantification) could be enhanced. In the HPLC separation, the recently introduced Kinetex phenyl-hexyl core–shell type column was used that enabled baseline separation for dexamethasone and its β-epimer, betamethasone. Dexamethasone and betamethasone were eluted within 12 min and such reduced retention, obtained with core–shell HPLC type column, further enhanced the sensitivity. The method was validated according to the European Union (EU) 2002/657/EC Decision; the studied parameters met the EU standards. The decision limits and limit of detections were calculated in each matrix for all corticosteroids and varied from 0.01 to 13.3 μg/kg and from 0.01 to 0.1 μg/kg, respectively.

1. Introduction

Endogenous corticosteroids are produced by the adrenal cortex (e.g. cortisol) and have important effects on a variety of metabolic events, including glucose and protein metabolism. The overall effect is to increase the blood glucose level by stimulating hepatic synthesis of glucose from amino acids [1]. Nowadays, several exogenous corticosteroids (prednisolone, dexamethasone, betamethasone, methylprednisolone) are authorized for therapy in both human and veterinary practices. They affect glucose utilization, fat metabolism, and bone development and are commonly used in the treatment of allergic reactions. They are also used to reduce inflammation [1–3]. Exogenous corticosteroids, however, suppress the body's natural production of corticosteroids by inhibiting the release of the hormone, adrenorticotropic. In the EU, the application of corticosteroids for food-producing animal therapy has been controlled, because corticosteroids have growth promoter properties, too [1–3]. Therefore, a minimum required performance limit (MRPL) concentration of 2 μg/L has been set for dexamethasone in urine by EU [4]. In edible tissues, and milk, however, maximum residue limits (MRLs) have been established for prednisolone, dexamethasone, betamethasone, and methylprednisolone (Table 1), which is regulated in Commission Regulation (EU) No 37/2010 [5]. Prednisone, methylprednisone, flumethasone and triamcinolone acetonid are non-authorized and no MRLs have been set for them. However, we set MRLs for these compounds in the present study in order to obtain concentration levels for validating the method. The selection of established MRLs for prednisone and methylprednisone was based on the official MRLs of prednisolone and methylprednisolone, respectively. We also established the same levels (5 μg/kg) for flumethasone and triamcinolone acetonid in all matrices, which could be easily detected

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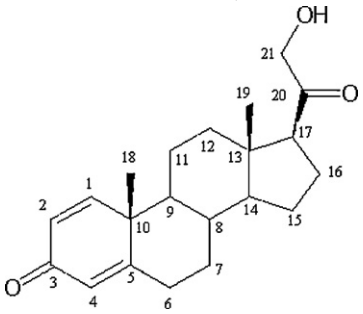
Table 1
Structures of corticosteroids, and MRLs for tissues in EU. The chosen MRLs for non-authorized compounds (PREDON, METPREDON, FLU, and TRIAM-AC).



Corticosteroids	Abbreviation	C6	C9	C11	C16	C16–17	C17	Animal species	Target tissues	MRLs (μg/kg)
Prednisolone	PRED			—OH			—OH	Bovine	Muscle	4
									Liver, kidney	10
Prednisone	PREDON			=O			—OH	Bovine	Muscle	4
									Liver, kidney	10
Dexamethasone	DXM		—F	—OH	—CH_3 (α)		—OH	Bovine, porcine, equidae	Muscle	0.75
									Liver	2
									Kidney	0.75
Betamethasone	BTM		—F	—OH	—CH_3 (β)		—OH	Bovine, porcine, equidae	Muscle	0.75
									Liver	2
									Kidney	0.75
Methylprednisolone	METPRED	—CH_3		—OH			—OH	Bovine	Muscle, liver, kidney	10
Methylprednisone	METPREDON	—CH_3		=O			—OH	Bovine	Muscle, liver, kidney	10
Flumethasone	FLU	—F	—F	—OH	—CH_3		—OH	Bovine	Muscle, liver, kidney	5
Triamcinolone acetonid	TRIAM-AC		—F	—OH		—O—$C(CH_3)_2$—O—		Bovine	Muscle, liver, kidney	5

MRLs for PREDON, METPREDON, FLU and TRIAM-AC applied in this study are not official regulated.

with the optimized method. It should be pointed out that the chosen MRLs for non-authorized compounds are not regulated; our aim was using the limits only for the validation. In the past, methylprednisolone was banned for the treatment of milk-producing animals, but now it has an MRL of 2 μg/kg in milk [6], which makes a higher possibility for the application of this veterinary drug. Another update regarding corticosteroids is the naturally production of prednisolone from cortisol in animals [7]. Hence, there is a need to develop analytical methods for the determination of corticosteroids in food and environmental samples to protect consumers from the harmful effects of residues.

Liquid chromatographic (LC) methods have been proven to be the most suitable techniques in corticosteroid analysis [1]. Although gas chromatographic (GC) separation can also be used for corticosteroid separation; it requires a time-consuming derivatization step [8,9] that makes it less preferable. Due to the complexity of biological matrices, LC-DAD (diode array detector) methods have not enough selectivity for robust determination. Additionally, the EU does not allow the confirmation of banned substances using DAD [1]. LC–MS/MS method is known as the most suitable for the determination of corticosteroids in different complex biological matrixes [1,10–14]. It has been recognized in LC–MS practice that the determinations need careful sample preparation in order to avoid the matrix effects (ion suppression and enhancement) in the ion source. Consequently, the main goal of sample preparation for LC–MS analysis is to reduce the number and concentration of matrix compounds as much as possible to avoid the above mentioned effects [10–14].

Recently, we have demonstrated the importance of sample pH during the application of mixed-mode SPE cartridges in the clean-up of urine and milk samples, which were prepared for corticosteroid analysis [10,13]. It must be mentioned that the selection of mixed-mode SPE cartridges and the sample pH used in clean-up procedure depend on biological matrices. Determination of corticosteroids in milk samples, the acidic pH control and mixed-mode cation exchange cartridges gave the most acceptable results [10], while for urine samples the basic pH and mixed-mode anion exchange SPE columns must be selected [13]. Based on our earlier results we have continued the research effort in this paper, how the pH influence the goodness of sample preparation of bovine tissues (muscle, liver, and kidney). To get a clear picture about the effect of sample pH, both acidic and basic pH were tested, and the results were evaluated. Additionally, the neutral condition, which included the application of sample pH of 7 and polymeric reversed-phase SPE column, was also investigated. The matrix effect of LC–MS/MS analysis is also studied according to Matuszewski et al. [15]. To the best of our knowledge only one method has been known in the literature that was developed for three tissues [16], however, this method was optimized for the analysis of only dexamethasone. In the present paper, eight corticosteroids were investigated in bovine muscle, liver and kidney, while in other existing LC–MS methods include the analysis of multi-compounds in one [17–23] or maximum of two tissues [24,25] without applying pH control. Another challenge in corticosteroid analysis is the simultaneous determination of dexamethasone and its β-epimer, betamethasone. Both substances are authorized and have MRLs in tissues, and therefore, the determination of these epimers is required. Previous studies mentioned above were successful in separation of dexamethasone and betamethasone using HPLC or UPLC determination and porous graphite, proteo or fully porous C-18 columns [17–25]. The other novelty of this determination is the application of the recently introduced Kinetex phenyl-hexyl core–shell type HPLC column that

was used for the simultaneous separation of epimers and other corticosteroids. This HPLC column has not been tested and applied for corticosteroid analysis yet.

This paper reports an LC–MS/MS method for the determination of eight corticosteroids. During the method development the sample preparation, and subsequent LC–MS/MS separation were optimized to reduce the matrix effects during the MS/MS analysis and to achieve as low analytical limits as possible for the chosen steroids. Finally, the developed method was validated for all matrices in line with EU 2002/657/EC Decision [26], and the results obtained from the validation met the EU guideline.

2. Experimental

2.1. Reagents, samples, equipments and instruments

The studied corticosteroids: prednisolone (PRED), prednisone (PREDON), dexamethasone (DXM), betamethasone (BTM), methylprednisolone (METPRED), flumethasone (FLU) and triamcinolone acetonid (TRIAM-AC) were purchased from Sigma–Aldrich (Budapest, Hungary). 1 mg/mL stock solution of methylprednisone (METPREDON) in ethanol was obtained from European Union Reference Laboratory (Wageningen, The Netherlands). The cortisol-d4 was obtained from Sigma–Aldrich (Budapest, Hungary) and used as internal standard.

Stock solutions were individually prepared by dissolving 10 mg of standards into 10.0 mL of methanol to obtain 1 mg/mL concentration. These solutions were stored at −20 °C for up to one year [10]. Three working standard solutions (one for muscle, one for liver, and one for kidney) were prepared weekly in methanol and were stored at 4 °C. The concentration of standards varied in working standard solutions, depending on the MRL of target compounds in the investigated matrices. Methanol and sodium acetate were of HPLC grade and were obtained from Merck (Budapest, Hungary). Ammonium acetate (99.999%) was obtained from Sigma–Aldrich (Budapest, Hungary). Ultrapure acetic acid (100%), formic acid (98–100%) and ammonia solution (25%) were purchased from Merck (Budapest, Hungary). A 2 M acetate buffer (pH 5.2) was prepared by dissolving 129.5 g sodium acetate in 800 mL water and adding 25.2 g acetic acid. After adjusting the pH to 5.2 ± 0.1, water was added to achieve a final volume of 1 L. The pH was checked and found to be 5.2. 1 MU *Helix Pomatia* β-glucuronidase/aryl sulfatase was purchased from Calbiochem (San Diego, CA, USA). Phenomenex Strata SPE cartridges (3 mL, 200 mg, 100 μm) for the sample cleaning procedure and Phenomenex Kinetex core–shell type HPLC columns for chromatographic separations were purchased from Gen-lab Ltd. (Budapest, Hungary). Phenex nylon membrane filters (0.45 μm) and HPLC vials were also purchased from Gen-lab Ltd. (Budapest, Hungary). The bovine tissue samples, originated from the Hungarian residue control monitoring program, were collected from July 2011 to February 2012 and were stored at −20 °C until subjected to analysis. A bovine liver control material (CM) containing incurred dexamethasone (assigned value: 3.41 μg/kg) was obtained from TEST VERITAS S.r.l. (Padova, Italy).

The centrifuge applied during the sample preparation was a Sigma 3-18K (Osterode am Harz, Germany), and the shaker was a Janke & Kunkel IKA KS125 shaker (Staufen, Germany). Sample evaporator was a TurboVap LV (Hopkinton, MA, USA). The LC–MS/MS (triple quadrupole) system was Agilent 6410A Triple Quad LC/MS (Agilent Technologies, Palo Alto, CA, USA) equipped with Agilent 1200 binary pump HPLC and Agilent 6410A mass selective detector. The interface was an Agilent multimode ion source (MMI). Data acquisition was performed using Agilent Mass Hunter B 01.04. Acquisition software, the data evaluation was performed on Agilent Mass Hunter B 01.03. Qualitative and Agilent Mass Hunter B 01.04. Quantitative software.

2.2. Sample hydrolysis and preparation

The enzymatic hydrolysis regarding the pH of sample, hydrolysis time, and temperature was carried out using a previously optimized procedure [19]. Initially, 2.5 g minced tissues were weighed into 50 mL polypropylene (PP) centrifuge tubes. Then 5 mL of 2 M acetate buffer (pH 5.2) and 10 μL *Helix Pomatia* juice were added to the samples, followed by vortex-mixing for 10 s. Samples were hydrolyzed for 4 h at 40 °C.

After the hydrolysis samples were cooled down at ambient temperature to 25 °C, they were subjected to solid–liquid extraction (SLE), and the extracts were further cleaned-up with solid-phase extraction using polymeric Strata SPE cartridges. The internal standard (5 μg/kg cortisol-d4) was added to the samples after the SPE procedure. The sample preparation conditions are summarized in Table 2.

2.2.1. Sample extraction and clean-up using acidic pH control (acidic condition)

4 mL of ethyl acetate–formic acid (98/2, v/v) extraction solution was added to the hydrolyzed samples. Samples were vortex-mixed for 30 s and shaken at 700 min^{-1} for 30 min at 25 °C. Samples were then centrifuged at 10,000 rpm for 10 min at 15 °C. Supernatant organic layers were transferred into glass tubes. Extraction was repeated one more time and organic upper layers were united in the tubes. 2 mL of 2% aqueous formic acid solution (v/v, pH 2.3) was added to the tubes, and tubes were vortex-mixed for 5 s. Samples were evaporated under a gentle nitrogen stream at 40 °C till the organic phase evaporated (∼1.5 mL), then they were let to cool down at ambient temperature to 30–35 °C. The volume of samples was adjusted to 3 mL with water containing 2% formic acid and they were re-dissolved by vortex-mixing for 30 s. Extracted samples were cleaned-up on Strata-XL-C (3 mL, 200 mg) SPE cartridges, which were previously conditioned two times with 3 mL methanol, followed by two times with 3 mL water, and finally with 3 mL water containing 2% formic acid. Samples were loaded to the SPE columns and passed through drop wise. Then cartridges were rinsed with 6 mL water containing 2% formic acid (in two aliquots) before drying the columns with vacuum for 2 min. Samples were eluted two times with 2.5 mL ethyl acetate containing 2% (v/v) formic acid into glass receiving tubes. Samples were evaporated to dryness under a gentle nitrogen stream at 45 °C and re-dissolved in 0.5 mL methanol–water (50/50, v/v) mixture by vortex-mixing for 30 s. Finally, samples were filtered through nylon filters into HPLC vials.

2.2.2. Sample extraction and clean-up using neutral and basic conditions

Samples were extracted and cleaned-up using the same procedure as was followed for the acidic condition, but other solvent compositions and SPE cartridges were applied. Neutral and basic conditions were carried out using solvents without pH control and solvents with basic pH control, respectively. In the case of neutral condition Strata-XL SPE cartridges were used for solid-phase extraction, while Strata-XL-A SPE columns were applied for basic condition. Conditions are summarized in Table 2.

2.3. LC–MS/MS conditions

Corticosteroids were separated in gradient elution mode on a Kinetex pheny-hexyl (100 mm × 4.6 mm, 2.6 μm) column equipped with a Phenomenex UHPLC phenyl guard column (3 mm × 4.6 mm). In the gradient program, two solvents (A and B) were mixed. Solvent

Table 2
Sample preparation conditions.

	Acidic condition	Neutral condition	Basic condition
Extraction solvent	4 mL ethyl acetate containing 2% (v/v) formic acid	4 mL ethyl acetate	4 mL ethyl acetate containing 2% (v/v) ammonia solution
Reconstitution solvent	3 mL water containing 2% (v/v) formic acid (pH 2.3)	3 mL water	3 mL water containing 2% (v/v) ammonia solution (pH 11)
SPE cartridge	Strata-XL-C	Strata-XL	Strata-XL-A
Condition solvent	Methanol, water, water containing 2% (v/v) formic acid (pH 2.3)	Methanol, water	Methanol, water, water containing 2% (v/v) ammonia solution (pH 11)
Washing solvent	Water containing 2% (v/v) formic acid (pH 2.3)	Water	Water containing 2% (v/v) ammonia solution (pH 11)
Elution solvent	2.5 mL ethyl acetate containing 2% (v/v) formic acid	2.5 mL ethyl acetate	2.5 mL ethyl acetate containing 2% (v/v) ammonia solution
Reconstitution solvent	0.5 mL methanol–water (50/50, v/v)	0.5 mL methanol–water (50/50, v/v)	0.5 mL methanol–water (50/50, v/v)

A contained 5 mM ammonium acetate and 0.01% (v/v) acetic acid in water (pH 5.4), solvent B was 100% methanol. Gradient elution started with 50% (v/v) B that was held for 12 min (isocratic section). Then solvent B increased from 50 to 100% (v/v) over 5 min, followed by 100% (v/v) B for 3 min. After 20 min, solvent B decreased to 50% (v/v) over 0.5 min. The flow rate was 0.8 mL/min and the total analysis time was 24.5 min. The injection volume was 10 μL and the column thermostat was set at 30 °C.

The mass selective detector was used in the multiple reaction monitoring (MRM) mode, and two ion transitions were set for a compound (Table 3). The more intense ion trace was used for quantification and the less intense one for qualification. The multimode ion source (MMI) operated in ESI mode with negative polarization. Nitrogen was used for drying and collision gas. The MMI settings were as follows: drying gas temperature: 300 °C; drying gas flow: 5 L/min; vaporizer: 160 °C; nebulizer pressure: 413.7 kPa (60 psi); capillary voltage: 2000 V; charging voltage: 2000 V. 500 V of delta electron multiplier voltage (ΔEMV) was set for the ion transitions of corticosteroids. This voltage enhances the secondary electron emissions in the photomultiplier, and consequently, the signal responses increase.

2.4. Quantification

Six point matrix-matched curves (including zero) were prepared by fortifying blank samples with different volumes of working standard solution at the beginning of sample preparation for compensating the losses during extractions. Calibration samples were extracted and cleaned-up as written above. In the case of PRED and PREDON, internal standard method was applied to evaluate the results. An ISTD (cortisol-d4) in 5 μg/kg concentration was used, which was added to the samples at the end of sample preparation to calibrate the ion source response. Unfortunately, cortisol-d4 was usable only for the above mentioned compounds. Other compounds eluted from the HPLC column at various time windows because of the differences in the polarity of selected corticosteroids. Therefore, the compounds were eluted along with other matrices. Furthermore, because of different background, cortisol-d4 was unusable in order to compensate the ion source for compounds eluted in other time window. However, in our study on the method development, we evaluated the results of other steroids using an ISTD method as well, but false values were obtained. Consequently, external standard (ESTD) method was applied for other compounds.

2.5. Evaluation of matrix effect

Matrix effect (ME) was studied for all sample preparation conditions written in Section 2.3. Five blank samples, which originated from different animals, for each matrix were extracted and cleaned-up. Samples were fortified with working standard solution to MRL values for all compounds after the clean-up procedure. Samples were analyzed and peak areas were integrated. A standard solution, which contained all studied corticosteroid in MRL concentration, was also prepared in HPLC clear methanol–water mixture (neat solution). This standard solution was also analyzed and signal responses were integrated. Absolute ME was calculated at MRL level as ME (%) = [(peak area of corticosteroid in matrix-matched solution/peak area of corticosteroid in neat solution − 1) × 100] [10,12,15]. Negative ME (<0) shows ion suppression, positive ME (>0) means ion enhancement. The relative ME was evaluated for all matrices at MRL level as the relative standard deviation (RSD%) of absolute matrix effects obtained from the five samples [10,12,15].

2.6. Validation

The developed method was validated in accordance with the EU 2002/657/EC Decision [26]. The analytical parameters were: selectivity, identification, linearity, recovery, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ), limit of detection (LOD), and limit of quantification (LOQ). Selectivity was investigated by comparing blank and spiked chromatograms. The identification was based on the ion ratios. In the case of linearity, the determination coefficient (r^2) was studied in the calibration ranges. For recovery, blank samples were spiked to 0.5 MRL, MRL and 1.5 MRL concentrations in six parallel for all compounds. Samples were analyzed and recovery (%) was calculated as (100 × measured concentration/spiking concentration). Within-laboratory reproducibility was investigated by repeating the recovery study on three different days. Between the three days different operators prepared the samples, different lots of SPE cartridges and solvents were used. Within-laboratory reproducibility was evaluated as the relative standard deviation (RSD%) of detected concentrations at each level [26]. In the case of compounds for which official regulated MRL has been set (PRED, DXM, BTM and METPRED), the CCα was calculated as MRL + 1.64 times the standard deviation of within-laboratory reproducibility at MRL level. For other compounds (PREDON, METPREDON, FLU and TRIAM-AC) three times of signal-to-noise ratio (SNR) was the estimated decision limit [26]. The CCβ was calculated as the decision limit plus 1.64 times the standard deviation of the decision limit. LOD and LOQ were calculated as 3 times and 10 times of SNR, respectively.

3. Results and discussion

3.1. Development of LC–MS/MS separation

The optimization of ion transitions of corticosteroids in Agilent 6410A MS/MS detector equipped with MMI is written in our recent studies [10–13], and these settings were applied in the present study. Corticosteroids can be ionized using either APCI or ESI sources [1]. Applying MMI the APCI mode resulted in higher

Table 3
MS/MS detector settings and ion ratio of two ion transitions of the analytes in standard solutions and spiked samples. Scan type: MRM; ion polarity: negative; ion mode: ESI.

Segment	Time (min)	ΔEMV	Compound	Precursor ion	Product ions	Dwell time (ms)	Fragmentor (V)	CE (V)	Ion ratios of standard solutions	Maximum permitted tolerances	Ion ratios of spiked samples
1	0–6.5	0	Data not stored								
2	6.5–8.5	500	PRED	$[M+CH_3COO]^-$ 419.3	**295.0** 280.1	100 100	110	25 25	94.7	75.8–113.6	89.9–106.4
			PREDON	$[M+CH_3COO]^-$ 417.1	**327.1** 299.0	100 100	90	0 20	18.5	13.0–24.1	17.0–18.9
			Cortisol-d4	$[M+CH_3COO]^-$ 425.3	**335.1**	100	90	15		ISTD	
3	8.5–11.1	50	Data not stored								
4	11.1–14.2	500	DXM	$[M+CH_3COO]^-$ 451.3	**361.1** 307.2	50 50	90	15 30	44.4	33.0–55.5	36.9–49.9
			BTM	$[M+CH_3COO]^-$ 451.3	**361.1** 307.2	50 50	90	15 30	32.8	24.6–41.0	28.1–37.6
			METPRED	$[M+CH_3COO]^-$ 433.2	**343.2** 309.2	90 90	110	15 25	18.3	12.8–23.8	17.7–18.6
			METPREDON	$[M+CH_3COO]^-$ 431.2	**342.2** 313.2	90 90	90	5 15	20.7	15.5–25.9	19.9–20.5
			FLU	$[M+CH_3COO]^-$ 469.3	**379.2** 305.3	90 90	140	10 30	13.4	9.4–17.4	13.3–17.7
5	14.2–15.0	50	Data not stored								
6	15.0–16.5	500	TRIAM-AC	$[M+CH_3COO]^-$ 493.3	**413.3** 337.0	200 200	150	20 20	31.9	23.9–39.9	31.9–33.8
7	16.5–24.5	0	Data not stored								

responses in analyzing urine, fat, milk or water samples for corticosteroids [10–13]. In these earlier studies, however, all steroids eluted from the HPLC column with an eluent containing higher level (>50%) of methanol. In the case of this tissue method, the more polar corticosteroids (PRED, PREDON, DXM and BTM) eluted with a mobile phase containing 50% methanol. This reduction in organic phase leaded to poorer responses for them in APCI mode. The application of higher vaporizer temperature was not recommended because the acetate adduct precursor ions can be broken at higher temperature, and consequently, less ions enter the MS/MS analyzer. Using the ESI part of MMI, an improvement was observed for these compounds and other steroids could also be measured with similar good sensitivity. We therefore used the ESI interface of MMI. Significantly, the temperature settings adapted from APCI optimization [10–13] are supported to ESI source by Agilent [27]. The dwell times were chosen to build up a peak from approximately 15 points, which is optimal for integration and reproducibility. For DXM and BTM, however, the application of a lower dwell time (50 ms) was necessary for the ion traces to get good peak shapes (Table 3). Consequently, the ion transitions of these two compounds were characterized with using approximately 25 points.

We used a core–shell type column for the HPLC separation to achieve high resolution, and consequently, to get baseline separation between DXM and BTM. These epimers differ only in the conformation of methyl group at C16 position (Table 1), so they have the same ion transitions, and therefore, the MS/MS analyzer cannot separate them. Due to this similarity in structures, the HPLC separation of epimers is difficult with conventional columns, however, they can be simultaneously determined within 7 min using columns packed with sub 2 μm particles and HPLC or UHPLC techniques [14,17]. For milk and fat samples we have demonstrated the effectiveness of a core–shell type HPLC column (Fused-Core® Ascentis Express C-18) in corticosteroid analysis regarding its improved sensitivity and better selectivity [10,12,28]. Initially, a C-18 phase core–shell column (Kinetex XB C-18) was tested for the separation of epimers that did not result in baseline separation. To enhance the resolution between DXM and BTM a phenyl-hexyl column was then used, which enabled improved separation for aromatic hydrocarbons. Kinetex pheny-hexyl HPLC column is the latest version of Kinetex family and has not been used for corticosteroid separation yet. A selectivity factor (α) of 1.05 could be achieved for DXM and BTM within 12 min using isocratic separation and methanol–acetate buffer (50/50, v/v) mobile phase. This mobile phase composition was found to be optimal. In the isocratic section in which the mobile phase composition contained a higher level (>50%) of organic modifier, the baseline separation could not be achieved, while a lower level of methanol (<50%) increased the analysis time. After 12 min isocratic run, gradient elution was used with a linear gradient program (linear solvent strength, LSS) in order to elute non-polar steroids. In using acetonitrile instead of methanol in the mobile phase, the DXM and BTM could not be separated at the baseline.

3.2. Optimization of sample preparation

The aim of LC–MS sample preparation is to reduce the matrix effects (ion suppression/enhancement) in ion source, which influence both the sensitivity and the accuracy of the method [15]. Recently, we have demonstrated that the pH is one main parameter to reduce the matrix compounds during the sample clean-up [10,13,29,30]. The corticosteroids are non-polar and neutral molecules, however, matrix compounds generally can be ionized in the sample extracts depending on pH, therefore mixed-mode ion exchange SPE cartridges can be chosen for sample clean-up and concentration. These SPE columns posses both non-polar (reversed-phase) and strong ion exchange characters [10,13]. The

pH must be adjusted during the sample preparation to remove the most of matrix compounds. The proper adjustment of pH of the samples would help to remove the ionizable matrix compounds and to get relatively clean samples. Mixed-mode SPE clean-up enables the selective adsorption of neutral corticosteroids and ionic matrices of the sample to the reversed-phase and to the ion exchange phase of the cartridge, respectively. In this study polymeric Strata mixed-mode strong cation exchange (Strata-XL-C) and mixed-mode strong anion exchange (Strata-XL-A) cartridges were tested using acidic and basic pH control, respectively. In addition, Strata-XL cartridges, which are high capacity polymeric reversed-phase SPE columns, were also tested without applying pH control.

During the optimization of sample preparation, the absolute and relative matrix effects (ME) were studied using different extraction conditions. Details are given in Section 2.3. Firstly, we used the acidic conditions in the sample preparation. For muscle and liver samples the absolute ME was found from −4.5% to 15.0% (relative ME, RSD: 0.7–8.1%) and from −16.2% to −8% (relative ME RSD: 4.3–10.1%) for corticosteroids, respectively. For kidney the absolute ME ranged between −30.2% and −13.2% (RSD: 6.1–10.7%). When neutral condition was investigated the absolute ME in muscle samples gave high ion suppression −68.4%–(−62.1%) for all steroids. The relative ME was 17.3–23.7%. For liver and kidney samples the absolute ME varied from −44.1% to −18.5% (RSD: 5.1–11.2%) and from −17.2% to 8.8% (RSD: 4.0–8.1%), respectively. We could conclude that the matrix effect was higher in muscle and liver samples compared to acidic condition. For kidney, the ME was a little lower utilizing neutral condition. After investigating the acidic and neutral conditions, we adjusted the sample pH to basic (pH 11) to obtain a clear understanding the role of pH in determination of selected corticosteroids in tissue samples. In the case of muscle, the absolute ME was between −44.4% and −29.3% (RSD: 9.8–12.9%), which is also higher compared to the acidic condition. For liver and kidney matrices, the absolute ME was found from −18.9% to −4.1% (RSD: 3.1–7.8%) and from −14.6% to −1.6% (RSD: 6.5–10.7%), respectively. The final conclusion is that both acidic and basic conditions were better than neutral condition. The matrix effects for muscle were the best with acidic condition. In the case of liver samples, similar matrix effects could be achieved in utilizing acidic or basic control. For kidney samples, slightly better results were obtained using the basic condition compared to acidic one. Results given above highlighted the role of pH adjustment and mixed-mode SPE cartridges in sample preparation of corticosteroids in muscle, liver, and kidney samples.

In this study we applied polymeric SPE cartridges with different surface chemistry. The three SPE cartridges were the following: Strata-XL, Strata-XL-C and Strata-XL-A. The surface of Strata-XL, which is a copolymer of divinylbenze and N-vinylpyrrolidone, has both non-polar and polar parts. This sorbent does not provide adequate selectivity for ionic molecules, and consequently, the interaction with ionizable matrix compounds is about the same as with corticosteroids. This fact explains the co-elution of steroids and matrices. The surface of Strata-XL-C sample preparation cartridges contains both non-polar and strong cation exchange sites (benzenesulfonic acid groups). Using Strata-XL-C cartridges and adjusting the pH to acidic, the basic matrix compounds and neutral steroids would adsorb on cationic sites and on non-polar sites, respectively. Acidic matrix compounds have weak adsorption on these SPE columns, therefore, they can be removed with washing the SPE column prior to sample elution. Eluting the corticosteroids with acidic organic solvent the basic matrices cannot elute from the SPE column. The eluent contained the corticosteroids and no basic solutes. Strata-XL-A cartridges have both non-polar and strong anion exchange (quaternary ammonium cation groups) sites. Under basic conditions, the corticosteroids adsorbed on non-polar surface of the column, similar to Strata-XL-C, and the deprotonated acidic matrices interacted with strong anion exchange groups. The basic matrices could be removed during the column washing prior to sample elution. Using basic organic solvent for sample elution, the cartridges retained the acidic matrices, while neutral (non-polar) corticosteroids eluted from the SPE cartridges.

Finally, it can be concluded that both pH acidic and basic conditions with the corresponding mixed-mode cartridges could be applied. We have chosen the acidic control because the monitoring measurements focus on muscle and liver samples and less kidney samples are analyzed.

3.3. Selectivity

Five different blank samples for each matrix were analyzed. The chromatograms of fortified samples (Fig. 1) were compared to chromatograms of blank samples (Fig. 2). All ion transitions were free of matrix peaks except the PRED. The most intense ion transition of PRED is the 419.2 $m/z \gg$ 329.2 m/z [10–13]. This ion trace had a matrix peak at the same time window where PRED eluted. This matrix was also observed in urine samples, however, it could be separated from PRED using Gemini C-18 HPLC column [13]. Using the phenyl-hexyl column, the interference could not be avoided in tissues. Therefore, we applied another quantification ion trace for PRED (419.2 $m/z \gg$ 295.0 m/z). Significantly, the LOD of a compound depends on the qualification ion trace, and therefore, this modification did not influence the LOD of PRED. This ion transition had also a matrix peak, however, it can be separated from the PRED (Fig. 1). The chromatogram of blank sample (Fig. 2) shows that matrix peaks had no influence on elution of the investigated compounds. Consequently, the selectivity of the developed LC–MS/MS method met the guideline.

3.4. Identification

In the case of MS/MS detection, four identification points are required [26]. Two ion transitions of a compound fulfill this requirement because the precursor ion means 1 identification point and 1 ion transition means 1.5 points. Detection with one precursor ion and its two ion transitions resulted in four identification points for a molecule. For MS/MS detection, the identification is based on the ion ratio (IR), which is the intensity ratio of qualifier and quantifier ion traces. The average of IR of a compound was calculated from the standard solutions ($n = 5$) for all matrices, and the permitted tolerance ranges were set according to the EU standards [26]. The IR in spiked and incurred samples was in the acceptable range (Table 3), and consequently, the identification met the requirements.

3.5. Linearity

Six-point calibration was performed for all analytes at 0 μg/kg, 0.25 MRL, 0.5 MRL, MRL, 1.5 MRL and 2 MRL levels. Slopes, intercepts and determination coefficients (r^2) are summarized in Table 4. The determination coefficients were between 0.9744 and 0.9999.

3.6. Recovery, within-laboratory reproducibility

Recovery % was investigated for each matrix and calculated from 18 results per spiking level (3 days × 6 results at each level per matrix). According to EU 2002/657/EC decision the recoveries at 1–10 μg/kg and under 1 μg/kg levels must be 70–110% and 50–120%, respectively. The within-laboratory reproducibility (expressed in RSD%) has to be as low as possible at these levels [26]. Recovery % and within-laboratory reproducibility are summarized in Table 4. In the case of muscle matrix, the recoveries were

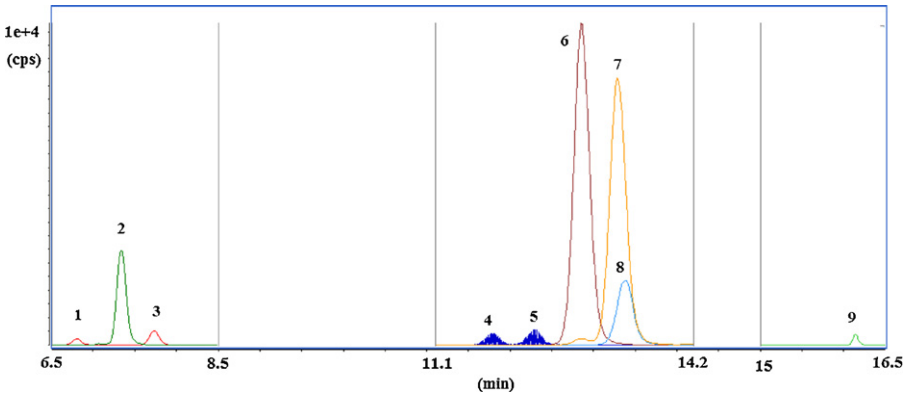


Fig. 1. Quantifier MRM chromatograms of corticosteroids in a spiked meat sample: 1. matrix; 2. PREDON (4 μg/kg); 3. PRED (4 μg/kg); 4. DXM (0.75 μg/kg); 5. BTM (0.75 μg/kg); 6. METPREDON (10 μg/kg); 7. METPRED (10 μg/kg); 8. FLU (5 μg/kg); 9. TRIAM-AC (5 μg/kg).

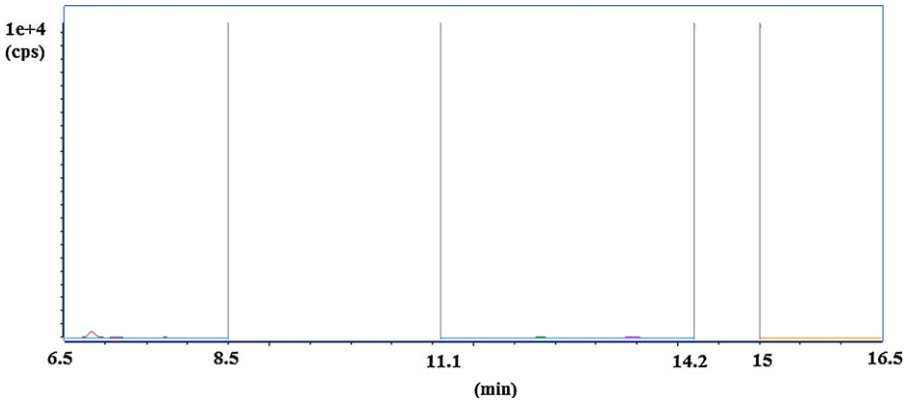


Fig. 2. Quantifier MRM chromatograms of corticosteroids in a blank meat sample.

Table 4
Validation results for all corticosteroids in each matrix. Linearity results in calibration ranges. Recovery% ranges between 0.5 MRL and 1.5 MRL levels. Within-laboratory reproducibility, and analytical limits.

	PRED	PREDON	DXM	BTM	METPRED	METPREDON	FLU	TRIAM-AC
Muscle matrix								
Slope	0.3737	2.3720	29,037	39,771	28,196	31,229	14,364	2030
Intercept	−0.0093	−0.0627	−336	−695	1443	4733	−2186	211
r^2	0.9791	0.9880	0.9995	0.9982	0.9999	0.9997	0.9802	0.9995
Recovery%	95–114	97–113	95–96	102–119	94–99	90–98	105–110	94–101
Reproducibility (RSD%)	6.5–14.6	6.0–15.5	6.7–12.5	2.5–16.7	5.1–11.1	6.3–12.4	3.4–6.6	4.0–11.1
CCα (μg/kg)	4.9	0.03	0.90	0.93	11.8	0.02	0.01	0.10
CCβ (μg/kg)	5.8	0.06	1.04	1.12	13.6	0.04	0.02	0.18
LOD (μg/kg)	0.03	0.03	0.02	0.01	0.02	0.02	0.01	0.10
LOQ (μg/kg)	0.10	0.10	0.07	0.03	0.07	0.07	0.03	0.33
Liver matrix								
Slope	0.103	0.627	36,620	31,649	24,074	23,356	10,384	1896
Intercept	0.013	0.014	−760	452	−1144	−603	−1053	−241
r^2	0.9799	0.9843	0.9852	0.9804	0.9928	0.9937	0.9902	0.9884
Recovery%	82–94	91–103	100–113	100–102	99–106	105–110	101–111	106–107
Reproducibility (RSD%)	10.1–12.1	7.3–12.7	4.0–8.3	6.9–15.7	2.3–3.6	2.2–3.3	3.6–4.5	3.3–4.5
CCα (μg/kg)	11.4	0.03	2.20	2.22	10.4	0.03	0.02	0.10
CCβ (μg/kg)	12.7	0.05	2.49	2.45	10.8	0.04	0.03	0.16
LOD (μg/kg)	0.03	0.03	0.03	0.02	0.03	0.03	0.02	0.10
LOQ (μg/kg)	0.10	0.10	0.10	0.07	0.10	0.10	0.07	0.33
Kidney matrix								
Slope	0.0536	0.351	33,467	42,176	27,648	31,420	13,377	1650
Intercept	0.0040	0.0068	406	929	11,721	7974	2031	−60
r^2	0.9910	0.9905	0.9862	0.9836	0.9878	0.9883	0.9744	0.9901
Recovery%	102–109	109–112	84–101	82–101	85–96	96–101	100–112	98–103
Reproducibility (RSD%)	9.7–22.1	4.7–18.7	19.4–25.8	18.0–27.6	12.3–23.1	8.7–20.3	8.6–22.3	6.0–12.3
CCα (μg/kg)	13.3	0.05	1.0	1.08	13.1	0.03	0.01	0.10
CCβ (μg/kg)	16.6	0.10	1.30	1.40	16.2	0.06	0.02	0.18
LOD (μg/kg)	0.05	0.05	0.03	0.03	0.03	0.03	0.01	0.10
LOQ (μg/kg)	0.17	0.17	0.10	0.10	0.10	0.10	0.03	0.33

Table 5
LC–MS methods for the determination of corticosteroids in edible tissues.

Compounds	Matrix	Clean-up	Technique	Column/mobile phase	Analytical limits (μg/kg)	Reference
DXM	Bovine muscle, liver, and kidney	SPE on C-18 phase	LC–APCI-MS^n, IT (positive ionization)	PLRP-S, polymeric RP AcN/water + 0.1% acetic acid	CCα: 1.2–2.2 LOQ: 0.38–1.0	Cherlet et al. [16]
PRED, BTM, DXM, METPRED	Liver from various species	SPE on C-18 and silica phase	UPLC–ESI-MS/MS (negative ionization)	Acquity BEH C-18, fully porous AcN + 0.5% acetic acid/water + 0.5% acetic acid	CCα: 2.31–11.93 LOD: 0.05–0.1	Deceuninck et al. [17]
DXM and BTM	Bovine liver	Only ASE	LC–APCI-MS/MS (negative ionization)	Kingsorb C-18, fully porous AcN/MeOH/water + 5 mM ammonium acetate	LOQ: 1.0	Draisci et al. [18]
11 corticosteroids including DXM and BTM	Bovine liver	SPE on C-18 phase	LC–ESI-MS/MS (negative ionization)	Hypercarb, porous graphite AcN/water + 0.3% formic acid	CCα: 0.08–10.11	Van den Hauwe et al. [19]
8 corticosteroids including DXM and BTM	Bovine liver	Only SLE using acetate buffer	LC–APCI-MS^n, IT (positive ionization)	PGC, porous graphite MeOH/DCM	LOQ: 0.33–40	Baiocchi et al. [20]
12 corticosteroids including DXM and BTM	Muscle	SPE on C-18, LLE, and SPE on silica phase	LC–ESI-MS/MS (negative ionization)	Nucleosil C-18 AB, fully porous MeOH/water + 0.5% acetic acid	LOD: 0.04–0.07	Antignac et al. [21]
9 corticosteroids including DXM and BTM	Bovine liver	SPE on HLB phase	LC–ESI-MS/MS (negative ionization)	Xterra MS C-18, fully porous AcN/water + 0.1% acetic acid	CCα: 2.15–10.8	Dusi et al. [22]
12 corticosteroids including DXM and BTM	Bovine liver	LLE and SPE on C-18 phase	LC–ESI-MS^n, IT (negative ionization)	Hypercarb, porous graphite AcN/water + 0.2% acetic acid	CCα: 0.43–12.6	Croes et al. [23]
11 corticosteroids including DXM and BTM	Bovine muscle and kidney	SPE on C-18 and MAX phase	UPLC–ESI-MS/MS (positive ionization)	Jupiter Proteo, fully porous AcN/water + 5 mM ammonium formate	LOQ: 0.4–1.6	Chrusch et al. [24]
8 corticosteroids including DXM and BTM	Meat and liver from various species	SPE on silica phase	LC–ESI-MS/MS (negative ionization)	Hypersil Gold C-18, fully porous AcN/water + 0.2% formic acid	LOQ: 0.5–4.0	Chen et al. [25]
8 corticosteroids including DXM and BTM	Bovine muscle, liver, and kidney	SPE on Strata-XL-C phase	LC–ESI-MS/MS (negative ionization)	Kinetex phenyl-hexyl, core–shell type MeOH/water + 5 mM ammonium acetate and 0.01% acetic acid	CCα: 0.01–13.3 LOD: 0.01–0.1 LOQ: 0.03–0.33	This study

AcN: acetonitrile; MeOH: methanol; DCM: dichloromethane; SLE: solid–liquid extraction; LLE: liquid–liquid extraction; ASE: accelerated solvent extraction; SPE: solid-phase extraction; MAX: mixed-mode anion exchange; IT: ion trap.

between 90 and 119% (RSD: 2.5–16.7%). The recoveries at 2 μg/kg level for PRED and PREDON were 114% and 113%, respectively. These results are a little higher than 110%. In the case of liver matrix, the recoveries ranged from 82 to 113%. For FLU 111% recovery was calculated at 2.5 μg/kg level that exceed with 1% the range set by EU. The reproducibility was 2.2–15.7%, which is acceptable at these levels. In the case of kidney matrix, the results were not as good as for other matrices, mainly, the reproducibility decreased. The higher matrix effects, which were obtained for kidney, could result in the lower results in the validation of kidney matrix. The recovery varied from 82% to 112%, and the reproducibility was between 4.7% and 27.6%. Reproducibility results can be accepted up to 30% at these levels [31].

3.7. Decision limit (CCα), detection capability (CCβ), limit of detection (LOD) and limit of quantification (LOQ)

Analytical limits were calculated for all corticosteroids in each matrix. CCα means a concentration of the compound at or above which the sample can be quantified as non-compliant with an error of α. For MRL substances (PRED, DXM, BTM and METPRED) this decision limit can be calculated from the corresponding MRL value and its reproducibility obtained from the validation (α = 5%) [26]. CCα was found between 0.90 and 11.8 μg/kg for muscle, ranged from 2.20 to 11.4 μg/kg for liver, and varied from 1.0 to 13.3 μg/kg for kidney matrix (Table 4). These results are similar to those calculated in previous studies (Table 5). In the case of substances for which no regulated MRL has been established (PREDON, METPREDON, FLU, and TRIAM-AC), the decision limit can be determined as 3 times of SNR (α = 1%) [26]. When MS/MS detection was used, the SNR had to be calculated for both ion transitions. The ion ratios must also be in the acceptable ranges at decision limit concentration. This approach required the analysis of twenty different blank samples from each matrix in order to calculate the SNR. For non-authorized compounds, CCα ranged from 0.01 to 0.10 μg/kg in different matrices (Table 4). The improved sample clean-up enabled excellent clear baseline for MRM transitions mainly in meat and liver samples, and therefore, the application of a higher delta electron multiplier voltage (ΔEMV) could be achieved for the ion traces (Table 3). This voltage is not able to enhance the SNR, if the ion transition is noisy, because it increases not only the signal response, but also the noise level. Consequently, the SNR is not changed. Since the ion transitions could be detected with decreased noise, the 500 V of ΔEMV improved the SNR that resulted in low CCα for PREDON, METPREDON, and FLU. Moreover, the minimized matrix effects ensured the reproducibility of ion ratios at low concentrations, too.

CCβ for MRL compounds means the content of a substance at which the method can detect permitted concentrations with an error of $1-\beta$. For PRED, DXM, BTM and METPRED (β = 5%) the detection capability varied from 1.04 to 13.6 μg/kg for muscle, ranged from 2.45 to 12.7 μg/kg for liver, and were between 1.30 and 16.6 μg/kg for kidney matrix (Table 4). In the case of non-authorized compounds, CCβ is the lowest concentration at which a method is able to detect contaminated samples with a certainty of $1-\beta$. For PREDON, METPREDON, FLU, and TRIAM-AC (β = 5%) detection capability was between 0.02 and 0.18 μg/kg. The CCα and CCβ values fulfilled the EU guidelines [26].

LOD varied between 0.01 and 0.03 μg/kg in muscle matrix for corticosteroids, except TRIAM-AC. In the case of TRIAM-AC, an LOD of 0.1 μg/kg was calculated in each matrix. LOD for other corticosteroids in liver and kidney matrices were ranged from 0.02 to 0.03 μg/kg and from 0.01 to 0.05 μg/kg, respectively. Limit of quantification ranged from 0.03 to 0.33 μg/kg for corticosteroids in different matrices (Table 4). The LOD and LOQ were also calculated from the SNR, so a reduction in these limits for PRED, DXM, BTM and METPRED could be achieved to those obtained in previous studies (Table 5). Limit of detections were confirmed by analyzing six blank samples for each matrix, which were spiked to the calculated LOD concentrations. LOD was accepted for a compound when the SNR in the fortified samples were higher than three and the ion ratios were in the acceptable range.

4. Analysis of real samples

4.1. Analysis of a control material

An incurred bovine liver CM was analyzed using the validated method. Three samples were prepared and analyzed in parallel. The detected concentrations were 1.63 μg/kg, 1.58 μg/kg, and 2.18 μg/kg, respectively. The mean value is 1.78 ± 0.35 μg/kg. According to the certificate of the sample the satisfactory range obtained from the proficiency test is between 0.85 and 5.97 μg/kg. The wide range suggests the difficulty of corticosteroid analysis in tissue samples. All of our three results were acceptable; consequently, the method showed good performance not only for spiked samples, but also for incurred ones.

5. Conclusions

A rapid LC–MS/MS method was developed for the confirmatory analysis of MRL regulated and non-authorized corticosteroids in bovine tissues. The sample clean-up was optimized to reduce ion suppression/enhancement effect of MS/MS analysis. In the sample clean-up, the application of acidic solvents and Strata-XL-C SPE cartridges could minimize the matrix effects in multimode ion source, and consequently, a reduction in analytical limits could be achieved. The HPLC separation on Kinetex phenyl-hexyl core–shell type column resulted in enhanced resolution and enabled baseline separation for dexamethasone and betamethasone. In the validation, the recovery of studied steroids was higher than 82% and the within-laboratory reproducibility were below 30%. The method allowed successful determination of dexamethasone in incurred liver sample.

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